

## Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates

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**ABSTRACT** Protein phosphorylation plays a central role in controlling many diverse signal transduction pathways in all cells. Novel protein kinases are identified at a rapid rate using homology cloning methods and genetic screens or selections; however identification of the direct substrates of kinases has proven elusive to genetic methods because of the tremendous redundancy and overlapping of substrate specificities among protein kinases. We describe the development of a protein engineering-based method to identify the direct substrates of the prototypical protein tyrosine kinase v-Src, which controls fibroblast transformation by the Rous sarcoma virus. To differentiate the substrates of v-Src from all other kinase substrates, we mutated the ATP binding site of v-Src such that the engineered v-Src uniquely accepted an ATP analog. We show that the engineered v-Src kinase displayed catalytic efficiency with the ATP analog, *N*<sup>6</sup>-(cyclopentyl) ATP, which is similar to the wild-type kinase catalytic efficiency with ATP itself. However, the *N*<sup>6</sup>-(cyclopentyl) ATP analog was not accepted by the wild-type kinase. Furthermore, the engineered v-Src exhibited the same protein target specificity as wild-type v-Src despite the proximity of the reengineered nucleotide binding site to the phosphoacceptor binding site. The successful engineering of v-Src's active site to accept a unique nucleotide analog provides a unique handle by which the direct substrates of one kinase (v-Src) can be traced in the presence of any number of cellular kinases.

Rous sarcoma virus transformation of fibroblasts is controlled by a single viral gene product, the protein tyrosine kinase v-Src (1). The rapid time course and dramatic morphological changes during fibroblast transformation by Rous sarcoma virus have made this system a paradigm for studies of oncogene activity in all cells. The origin (2), regulation (3–6), and structure (7–9) of v-Src have been studied extensively and are well characterized (3, 10, 11). Remarkably, the central question about this intensely studied kinase remains unanswered: what are its direct cellular substrates? Identification of the specific substrates for protein tyrosine kinases is difficult because the number of cellular kinases is enormous [it is estimated that 2% of the mammalian genome encodes protein kinases (12)] and because tyrosine kinases display overlapping substrate specificities (3, 13).

The expression of v-Src in fibroblasts results in the tyrosine phosphorylation of over 50 cellular proteins (10). These same substrates also are phosphorylated by other kinases in untransformed fibroblasts (14). Even the most sophisticated biochemical and genetic techniques (including anti-phosphotyrosine protein blots of transformed fibroblasts, transfection of fibroblasts with transformation-defective v-Src mutants, using temperature-sensitive v-Src mutants, gene knock-out studies of

cellular Src, using host range-dependent Src mutants, anti-v-Src immunoprecipitation, and use of kinase specific inhibitors) have not led to the unambiguous identification of v-Src's direct substrates (see ref. 11 for a comprehensive review). In fact, the direct substrates for the majority of cellular kinases remain unidentified (3).

We have developed a strategy to uniquely tag the direct substrates of the prototypical tyrosine kinase v-Src. Through protein engineering, we have created a structural distinction between the nucleotide binding site of v-Src and that of all other kinases. The use of a novel enzyme substrate or ligand that is orthogonal to the enzyme's naturally occurring target is a powerful tool for the study of single catalysts (15) and receptors (16) in multi-component enzyme systems. The v-Src kinase we have engineered recognizes an ATP analog (A\*TP), *N*<sup>6</sup>-(cyclopentyl) ATP, that is orthogonal to the nucleotide substrate of wild-type kinases. The generation of a v-Src mutant with specificity for an orthogonal A\*TP substrate allows for the direct substrates of v-Src to be uniquely radio-labeled because [ $\gamma$ -<sup>32</sup>P]*N*<sup>6</sup>-(cyclopentyl) ATP is recognized solely by the engineered v-Src kinase and no other cellular kinases.

### MATERIALS AND METHODS

**Synthesis of ATP Analogs.** Analogs 1, 2, 4, 6, 9, and 12 were synthesized via Dimroth rearrangement of the corresponding *N*<sup>1</sup>-alkoxy adenine derivatives prepared according to Fujii *et al.* (17). Analog 5 was synthesized via Dimroth rearrangement of *N*<sup>1</sup> benzyladenosine (18). Analog 3 was prepared according to McLaughlin *et al.* (19). Analogs 7, 8, 10, and 11 were synthesized via treatment of 6-chloropurine riboside (Aldrich) with pyrrolidine, cyclopentylamine, piperidine, and cyclohexylamine, respectively (20). Triphosphate synthesis was carried out according to the method of Ludwig (21) with the exception of the preparation of pyrophosphate. Accordingly, bis-tri-*N*-butyl ammonium pyrophosphate was prepared by mixing 1 equivalent of pyrophosphoric acid with 2 equivalents of tributyl amine in a 1:1 water-to-ethanol mixture until a homogenous solution was obtained. Solvent was removed under vacuum to dryness, and the pyrophosphate was stored over P<sub>2</sub>O<sub>5</sub> overnight. All nonradioactive nucleotides were characterized by <sup>1</sup>H-NMR, mass spectral analysis, and strong anion exchange-HPLC (Rainin Instruments model 83-E03-ETI). [ $\gamma$ -<sup>32</sup>P]*N*<sup>6</sup>-(cyclopentyl) ATP was synthesized according the method of Hecht and Kozarich (22). The radiolabeled analog was purified on DEAE (A-25) Sephadex, and the triphosphate was identified by co-injection of the radiolabeled material with an authentic sample of *N*<sup>6</sup>-(cyclopentyl) ATP on a strong anion exchange-HPLC column [Rainin Instruments; 5–750 mM

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Abbreviations: v-Src, Rous sarcoma virus tyrosine kinase; GST, glutathione *S*-transferase; XD4,  $\Delta(77-225)$  fragment of v-Src; RR-Src, peptide substrate for v-Src; SH, Src-homology; A\*TP, structural analog of adenosine triphosphate; PKA, cAMP-dependent kinase; cdk, cyclin-dependent kinase.

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ammonium phosphate (pH 3.9) in 10 min at 0.5 ml/min]. The chemical yield of the reaction varied from 70 to 80%.

**Protein Expression and Purification.** Overlap extension PCR was used to make glutathione *S*-transferase (GST)-XD4(V323A, I338A) (23). Pfu polymerase (Stratagene) was used in the PCRs according to the manufacturer's protocol. Six synthetic oligonucleotides were used: primer 1 (5'-TTTG-GATCCATGGGGAGTAGCAAGCAAG); primer 2 (5'-TTTGAATTCTACTCAGCGACTCCAACAC); primer 3 (5'-TGAGAAAGCTGGCTCAACTGTACGCAG); primer 4 (5'-CTGCGTACAGTTGAGCCAGCTTCTCA); primer 5 (5'-CTACATCGTCTGCTGAGTACATGAG); and primer 6 (5'-CTCATGTACTCAGCGACGATGTAG).

Primer 1 contains a *Bam*HI site, and primer 2 contains an *Eco*RI site (italics). Primers 3 and 4 contain the nucleotide sequence changes to introduce the V323A mutation (mutations shown in bold). Primers 5 and 6 contain the I338A mismatch. The XD4 gene from YEp51-XD4 plasmid (a gift of B. Cochran, Tufts Medical School) was amplified with primers 1 and 2. The PCR product was digested with *Bam*HI and *Eco*RI and ligated into similarly digested pGEX-KT and then transformed into the *Escherichia coli* strain DH5 $\alpha$ . The GST-XD4(V323A) was constructed using primers 1, 2, 3, and 4 with the GST-XD4 plasmid as the template. The resulting PCR product was digested with *Bam*HI and *Eco*RI, ligated into similarly digested pGEX-KT, and transformed into DH5 $\alpha$  *E. coli* cells. GST-XD4(V323A, I338A) was made in the same manner using primers 5 and 6 with GST-XD4(V323A) as the template. Expression and purification of kinases were carried out in DH5 $\alpha$  as described by Xu *et al.* (24), with the exception that the cells were stored at 4°C overnight before centrifugation and lysis by French press (overnight storage is essential for producing highly active kinases).

Expression of 6-His-XD4 and 6-His-XD4(V323A, I338A) in Sf9 insect cells was accomplished using the Life Technologies (Grand Island, NY) BAC-to-BAC system. The 6-His-XD4 and 6-His-XD4(V323A, I338A) genes were generated by PCR using the corresponding pGEX vectors as templates with primers 1 and 2, followed by digestion with *Bam*HI and *Eco*RI. The resulting PCR fragment was cloned into pFAST-BAC, which had been similarly digested. Transformation of HB101BAC cells and subsequent transfection of Sf9 cells with the bacmid-containing XD4 or XD4(V323A, I338A) were carried out exactly as suggested by the manufacturer.

**Protein Blots and Kinase Assays.** Assays of the nucleotide specificity of murine lymphocyte-derived protein kinases was performed using splenocytes (8- to 30-week-old male and female C57BL/6J (B6) mice from the Princeton University Animal Facility), which were isolated and washed in RPMI 1640 medium containing 5% bovine calf serum, 1% HEPES, and DNase I (1  $\mu$ g/ml). Red cells were lysed at 4°C by treatment with 17 mM Tris ammonium chloride (pH 7.2). The cells were lysed hypotonically on ice for 10 min in 1 mM HEPES (pH 7.4), 5 mM MgCl<sub>2</sub>, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, and 100  $\mu$ M of phenylmethylsulfonyl fluoride according to the method of Fukazawa *et al.* (25). After vortexing and centrifugation at 500  $\times$  g, the supernatant was collected. Cells were stored at 4°C for 20 min to attenuate the basal protein phosphorylation level, after which the buffer was adjusted to 20 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, and 1 mM NaF. Sodium vanadate (100  $\mu$ M) was then added to inhibit the activity of phosphotyrosine phosphatases. Each nucleotide was added to a final concentration of 100  $\mu$ M to 5  $\times$  10<sup>6</sup> cell equivalents and incubated at 37°C for 5 min, after which a 4X Laemmli gel loading buffer was added to quench the reaction. Proteins were separated by 12.5% SDS/PAGE and transferred to Protran BA85 (Schleicher & Schuell). The blot was probed with the anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY), and the bound antibody was detected via enhanced chemiluminescence after treatment with horserad-

ish peroxidase-coupled goat anti-mouse antibody (VWR Scientific 7101332). Similar anti-phosphotyrosine blots of Sf9 cells expressing 6-His-XD4 or 6-His-XD4(V323A, I338A) were carried out after lysis of 10<sup>6</sup> cells in a buffer containing 0.1% Triton X-100, 50 mM Tris (pH 8.0). Assays of serine/threonine as well as tyrosine kinases in the murine lymphocytes were performed similarly with the exception of the use of radiolabeled [ $\gamma$ -<sup>32</sup>P]ATP or [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP (5000 cpm/pmol) added to a final concentration of 100  $\mu$ M with 5  $\times$  10<sup>6</sup> cell equivalents and incubated at 37°C for 10 min, after which 4X Laemmli gel loading buffer was added to the cell lysate to quench the reaction. Proteins were separated by 12.5% SDS/PAGE. The gel was soaked in 10% acetic acid/10% isopropanol for 1 h, after which it was dried and exposed for 1 h.

Assays of GST-XD4 and GST-XD4(V323A, I338A) phosphorylation of the peptide substrate for v-Src (RR-Src) were carried out in triplicate at 37°C in a final volume of 30  $\mu$ l buffered at pH 8.0 containing 50 mM Tris, 10 mM MgCl<sub>2</sub>, 1.6 mM glutathione, 1 mg/ml BSA, 1 mM RR-Src peptide with either GST-XD4 (100 nM) or GST-XD4(V323A, I338A) (100 nM), and 10  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (1000 cpm/pmol) (DuPont/NEN). For inhibition studies, cold ATP or A\*TP analogs (100  $\mu$ M) (analog 1-12) were added before addition of the kinase. After 30 min, the reactions were quenched by spotting 25  $\mu$ l of the reaction volume onto p81 phosphocellulose disks (Whatman) and immersing in 250 ml of 10% acetic acid for >30 min, followed by washing and scintillation counting according to standard methods (26). Similar assays were used to measure *k*<sub>cat</sub> and *K*<sub>M</sub> values for the GST-kinases. Autophosphorylation of affinity-purified GST-XD4 (10 pmol) and GST-XD4(V323A, I338A) (10 pmol) were carried out in the presence of 50  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (5000 cpm/pmol) or [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP (5000 cpm/pmol) at 37°C for 1 h in a final volume of 30  $\mu$ l at pH 8.0, 50 mM Tris, and 10 mM MgCl<sub>2</sub> and loaded on a 12% SDS/PAGE gel for autoradiography as described for the lymphocyte assay.

## RESULTS AND DISCUSSION

**Protein Design Criteria.** We identified several criteria that would have to be satisfied in reengineering the v-Src kinase to uniquely tag its authentic substrates in the presence of wild-type tyrosine and serine/threonine kinases. The engineered v-Src protein would have to: (i) accept an ATP analog (A\*TP) that is a dead substrate for all wild-type protein

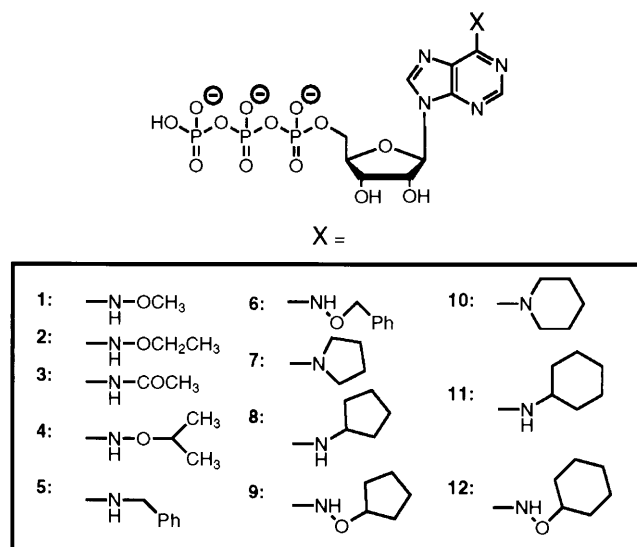


FIG. 1. Chemical structures of ATP analogs (A\*TPs) used to probe the nucleotide binding specificity of cellular protein tyrosine kinases.

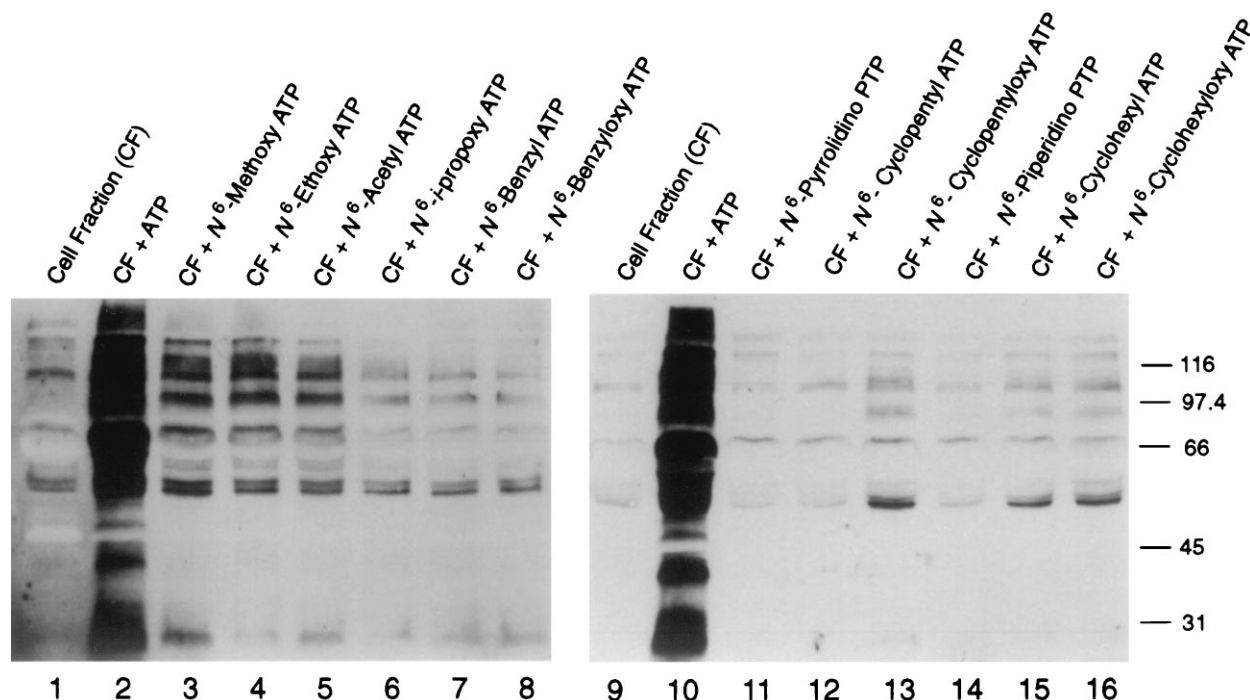
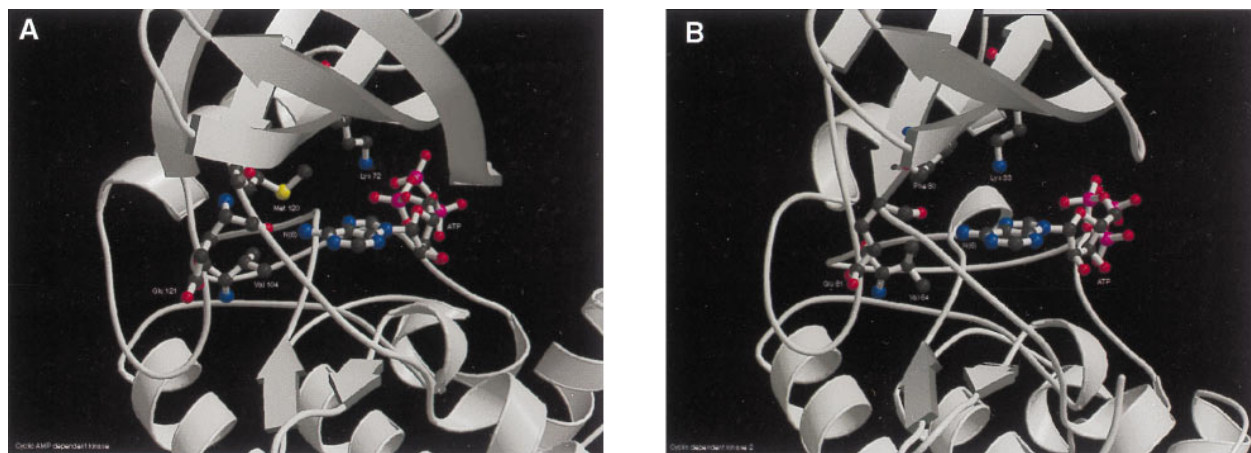


FIG. 2. Anti-phosphotyrosine protein immunoblot showing the level of protein tyrosine phosphorylation after treatment of a murine lymphocyte cell lysate (CF) with 100  $\mu$ M of ATP or A\*TPs (analog 1–12). This lysate includes Src, Fyn, Lck, Lyn, Yes, Fgr, Hck, Zap, Syk, Btk, Blk, and other tyrosine kinases present in B and T lymphocytes, macrophages, and follicular dendritic cells (28). Molecular size standards (in kilodaltons) are indicated.

kinases; (ii) use the A\*TP analog with high catalytic efficiency; and (iii) have reduced catalytic efficiency for the natural nucleotide substrate (ATP) so that, in the presence of cellular levels of ATP (1–2 mM), the mutated kinase would preferentially use A\*TP as the phosphodonor. All of

these criteria must be met without altering the protein target specificity of v-Src. The ATP binding site is very close to the peptide binding site, so it was not obvious when we initiated this work that it would be possible to satisfy all of these requirements simultaneously.



C	Subdomain	IV	V
	cAMP-Dependent Kinase	(99)NFPFL <b>V</b> KLEFSFKDNSNLYM <b>V</b> MEYVPG(125)	
	CDK2	(59)NHPN <b>I</b> VKLLDVIHTENKLYLV <b>F</b> FEFLHQ(85)	
	v-Src	(318)RHEKL <b>V</b> QLYAVVSE-EPIYIV <b>I</b> EYMSK(343)	

FIG. 3. (A) A close-up view of the ATP binding site in cAMP-dependent protein kinase (1ATP). Three residues within a 4-Å sphere of the N<sup>6</sup> amine of ATP (Val 104, Met 120, and Glu 121) in 1ATP and the catalytically essential lysine residue (Lys 72) are shown in ball-and-stick representation. The remainder of the protein is shown in ribbon format. (B) cdk2-cyclin D complex (1cdk) cocrystal structure. The 1cdk structure is shown with bound ATP, the residues in the 5-Å sphere of the N<sup>6</sup> amine of ATP (Val 64, Phe 80, and Glu 81) and the catalytic lysine residue (Lys 33) are shown in ball-and-stick representation. A and B were created by feeding the output of MOLSCRIPT into the RASTER3D rendering program (35, 36). (C) Sequence alignment of the ATP binding regions of PKA, cdk2, and v-Src. The bold residues correspond to the amino acids with side chains in a 5-Å sphere of the N<sup>6</sup> amino group of kinase-bound ATP.

**Nucleotide Specificity of Cellular Kinases.** To identify compounds that would not be accepted as substrates by any existing cellular kinases (27), we screened a panel of synthetic A\*TP analogs (Fig. 1, analogs 1–12) in a murine lymphocyte cell fraction rich in protein tyrosine kinases (Fig. 2) (28). The A\*TPs containing the smallest  $N^6$  substituents, 1 (methoxy), 2 (ethoxy), and 3 (acetyl), showed some ability to serve as cellular tyrosine kinase substrates (Fig. 2, lanes 3–5). The A\*TPs with sterically demanding  $N^6$  substituents, 4 (*i*-propoxy), 5 (benzyl), and 6 (benzyloxy), and all analogs containing cyclic aliphatic substituents (analog 7–12) showed little or no protein phosphorylation (Fig. 2, lanes 6–8 and 11–16). To test for possible metathesis of orthogonal A\*TPs (analog 4–12) with cellular ADP to give A\*DP and ATP, we added 1 mM ADP to the cell lysate kinase reactions shown in Fig. 2. The pattern of phosphoproteins was the same (data not shown), indicating that no significant metathesis of A\*TP occurs in a complete cell lysate system. Analog 4–12 are dead substrates for wild-type tyrosine kinases and thus were chosen as the targets for reengineering the nucleotide binding site of v-Src.

**Mutations in the ATP Binding Site of v-Src.** No crystal structures of any tyrosine kinases in an active conformation have been solved to date (29, 30). However, two crystal structures of catalytically active serine/threonine kinases have been solved (31, 32). There is a high degree of functional homology between the serine/threonine and the tyrosine kinase catalytic domains as shown by affinity labeling of the identical catalytically active lysine residue in both kinase families [K72 in cAMP-dependent kinase (PKA), K295 in v-Src] (33, 34). Inspection of the PKA (31) and cyclin-dependent kinase 2 (cdk2)–cyclin A (32) crystal structures revealed two amino acid side chains within a 5-Å sphere of the  $N^6$  amino group of bound ATP: V104/M120 (PKA; Fig. 3A) and V64/F80 (cdk2, Fig. 3B) (37). Based on the functional similarity between kinases, we mutated positions V323 and I338 (V104/M120 in PKA and V64/F80 in cdk2) (Fig. 3C) in the v-Src catalytic domain [Src-homology 1 (SH1) domain] to alanine to create an additional “pocket” in the nucleotide binding site of v-Src to allow binding of one of the orthogonal A\*TPs (analog 4–12). We then expressed both the wild-type and the double alanine mutant of the v-Src catalytic domain (the XD4 fragment) as GST fusion proteins (GST–XD4) (38, 39) in *E. coli*, which is a good expression host because it lacks any endogenous tyrosine kinases. The XD4 fragment of v-Src contains an intact SH1 catalytic domain but lacks the noncatalytic regulatory SH3 and SH2 domains and exhibits higher specific activity than full length v-Src (Fig. 4A) (38). We confirmed that the GST–XD4 and GST–v-Src (containing functional SH3 and SH2 domains) display the same  $K_M$  values for ATP and RR-Src. GST–XD4 displays higher specific activity than GST–v-Src, so it was used in our studies. We found that the wild-type GST–XD4 kinase phosphorylated a well characterized peptide substrate of v-Src, RR-Src, with kinetics consistent with those in literature reports (40) (Table 1).

**Phosphoacceptor Specificity of Wild-Type and Mutated XD4.** The GST–XD4(V323A, I338A) double mutant phosphorylates the RR-Src peptide with only slightly lower catalytic activity than wild-type GST–XD4 (Table 1). As hoped, however, the specificity constant ( $k_{cat}/K_M$ ) for the double mutant with ATP was considerably lower than for the wild type. To determine whether the alanine mutations have any effect on the protein substrate specificity, we measured the  $K_M$  of both the wild-type and the mutant fusion proteins for the RR-Src peptide. At saturating concentrations of [ $\gamma$ - $^{32}$ P]ATP, the wild type and the mutant display the same  $K_M$  for RR-Src:  $2.6 \pm 0.9$  mM and  $3.1 \pm 0.9$  mM, respectively (40). This suggests that the alanine mutations in the ATP binding pocket, which is proximal to the adjacent phosphoacceptor binding site, do not affect the protein target specificity. In support of this, the engineered kinase phosphorylates the identical set of proteins that are phosphorylated by wild-type XD4 when each is expressed in Sf9 insect cells (Fig. 5A, lanes 2 and 3). The Sf9

insect cell system is a good host for expressing small amounts of tyrosine kinases because these cells contain most of the same machinery necessary to carry out posttranslational modifications to proteins, resulting in kinases that are more similar in activity to those found in mammalian cells. Furthermore, uninfected Sf9 cells lack endogenous tyrosine kinase activity (Fig. 5A, lane 1), and thus the phosphotyrosine-containing proteins in Fig. 5A, lanes 2 and 3, are substrates of the expressed 6–His–XD4 and mutant 6–His–XD4 kinases, respectively. Taken together, these data show that the peptide and protein specificities of the engineered kinase are virtually identical to that of the wild-type XD4 fragment of v-Src.

**Novel Nucleotide Specificity of Mutant v-Src.** We next evaluated the ability of  $N^6$ -substituted ATP analogs 1–12 to differentially inhibit wild-type and mutant kinase phosphory-

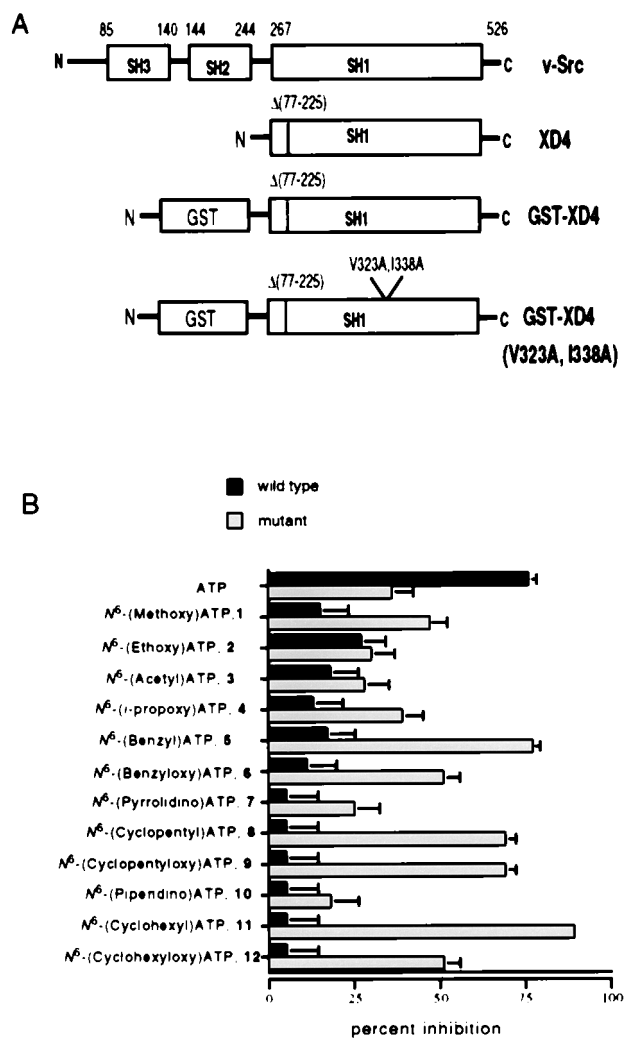


FIG. 4. (A) The domain structure of v-Src including the Src-homology 3, 2, and 1 (SH3, SH2, and SH1) domains with the domain boundaries indicated by the amino acid residues above each boxed domain. The domain structure of XD4 that contains a deletion of residues 77–225 ( $\Delta(77-225)$ ). Domain organization of the GST fusion with XD4 (numbering from v-Src) and the doubly mutated GST–XD4(V323A, I338A). (B) Relative inhibition of GST–XD4 (solid bars) and GST–XD4(V323A, I338A) (shaded bars) catalyzed RR-Src peptide phosphorylation by ATP and A\*TP analogs 1–12. Percentage inhibition ( $1-v_1/v_0$ ) is reported as a ratio of  $v_1$  [cpm in the presence of 100  $\mu$ M of the indicated triphosphate and 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (1000 cpm/pmol)]/ $v_0$  [cpm in the presence of 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (1000 cpm/pmol)] – background cpm due to nonspecific 10- $\mu$ M [ $\gamma$ - $^{32}$ P]ATP binding to the phosphocellulose disks (<0.1% of total input counts). Error bars represent the SD determined from four separate experiments with three replicates.

Table 1. GST-XD4 and GST-XD4(V323A, I338A) catalyzed RR-Src peptide phosphorylation kinetic constants

Nucleotide	GST-XD4			GST-XD4 (V323A, I338A)		
	$k_{cat}$ , min <sup>-1</sup>	$K_M$ , $\mu$ M	$k_{cat}/K_M$ , min <sup>-1</sup> ·M <sup>-1</sup>	$k_{cat}$ , min <sup>-1</sup>	$K_M$ , $\mu$ M	$k_{cat}/K_M$ , min <sup>-1</sup> ·M <sup>-1</sup>
ATP	2 ± 0.5	12 ± 3	1.6 × 10 <sup>5</sup>	0.8 ± 0.2	150 ± 20	5.3 × 10 <sup>3</sup>
N <sup>6</sup> -(cyclopentyl) ATP	>2000 (K <sub>i</sub> )			(5 ± 2) × 10 <sup>-2</sup>	15 ± 3	3.3 × 10 <sup>3</sup>

ATP and N<sup>6</sup>-(cyclopentyl) ATP-dependent RR-Src phosphorylation (1 mM) by GST-XD4(V323A, I338A) and GST-XD4 was measured at low substrate conversion (<5%) in triplicate. Kinetic constants were determined by analysis of Lineweaver-Burk plots of the rate data (41). Reactions were performed in the same manner as in Fig. 4B except for substitution of [ $\gamma$ -<sup>32</sup>P]ATP with [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP (5000 cpm/pmol) as indicated.

lation of RR-Src with [ $\gamma$ -<sup>32</sup>P]ATP (Fig. 4B). The wild-type kinase GST-XD4 displays poor binding affinity for most A\*TP analogs (Fig. 4B, solid bars), as expected from the lymphocyte kinase assay (Fig. 2). In contrast, the doubly mutated GST-XD4(V323A, I338A) shows excellent inhibition by more sterically demanding N<sup>6</sup>-substituted ATP analogs (Fig. 4B, shaded bars). Most significantly, the GST-XD4(V323A, I338A) mutant is inhibited by ATP analogs 5, 8, 9, and 11 as well as the wild-type kinase GST-XD4 is inhibited by its natural substrate ATP. We have confirmed that the GST-XD4(V323A, I338A) and the full length GST-v-Src (V323A, I338A) display the same inhibition pattern with A\*TPs (analog 1-12) (data not shown).

Four of the nine “dead” substrates identified in the screen of wild-type kinase specificity (Fig. 2) bind well to the mutant kinase. This high success rate in identifying new substrates for a mutant v-Src that are not accepted by wild-type kinases (i.e., orthogonal) suggests that we have identified a key feature of the v-Src nucleotide binding site, namely the residues that make a close fit around the N<sup>6</sup> amino group of ATP. It is worth noting that we know of no wild-type protein kinases that contain an alanine at the position corresponding to I338 in v-Src (position 120 in PKA). If a sterically demanding amino acid side chain at this position also plays a critical role in determining the nucleotide specificity of other kinases, it should be possible to engineer it to accept orthogonal substrates using an approach very similar to the one described here.

We chose to test the ability of N<sup>6</sup>-(cyclopentyl) ATP (analog 8) to serve as a catalytically competent substrate of both wild-type GST-XD4 and the GST-XD4(V323A, I338A) mutant over the other three ATP analogs (analog 5, 9, and 11) because analog 8 exhibited a slightly lower level of phosphorylation with wild-type kinases (Fig. 2, lane 12). The wild-type kinase GST-XD4 did not phosphorylate the RR-Src peptide with [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP, confirming our previous observations that this analog is not a substrate for the wild-type kinase. In contrast, GST-XD4(V323A, I338A) displayed Michaelis-Menten kinetics with the orthogonal A\*TP [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP (Table 1). The mutant kinase displays the same K<sub>M</sub> for the RR-Src peptide when [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP is the phosphodonor (K<sub>M</sub> = 2.0 ± 0.9 mM) instead of [ $\gamma$ -<sup>32</sup>P]ATP, confirming that the mutant kinase phosphorylates the same target with either ATP or A\*TP.

**Catalytic Efficiency of Mutant v-Src with N<sup>6</sup>-(Cyclopentyl) ATP.** The parameter used to rank catalysts for competing substrates is the measure of  $k_{cat}/K_M$  (the “specificity constant”) (41). The  $k_{cat}/K_M$  of the engineered mutant GST-XD4(V323A, I338A) with the orthogonal substrate [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP is only 50-fold lower than the  $k_{cat}/K_M$  of the wild-type kinase with its natural substrate, ATP. This catalytic efficiency with the orthogonal A\*TP substrate coupled with the drop in catalytic efficiency with ATP by the mutant kinase satisfies two of the critical design criteria we established at the outset. Even more significant, the new substrate [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP is orthogonal with respect to wild-type GST-XD4 as demonstrated by the complete inability of GST-XD4 to use this analog as a

phosphodonor for autophosphorylation (Fig. 5C, lane 3). In contrast, the engineered kinase is efficiently autophosphorylated with [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP (Fig. 5C, lane 4).

The ultimate goal of this work is to use mutant kinases specific for synthetic substrate analogs to tag direct kinase substrates in whole cells or cell lysates. This requires that no wild-type kinases, including serine/threonine-specific kinases, that carry out the

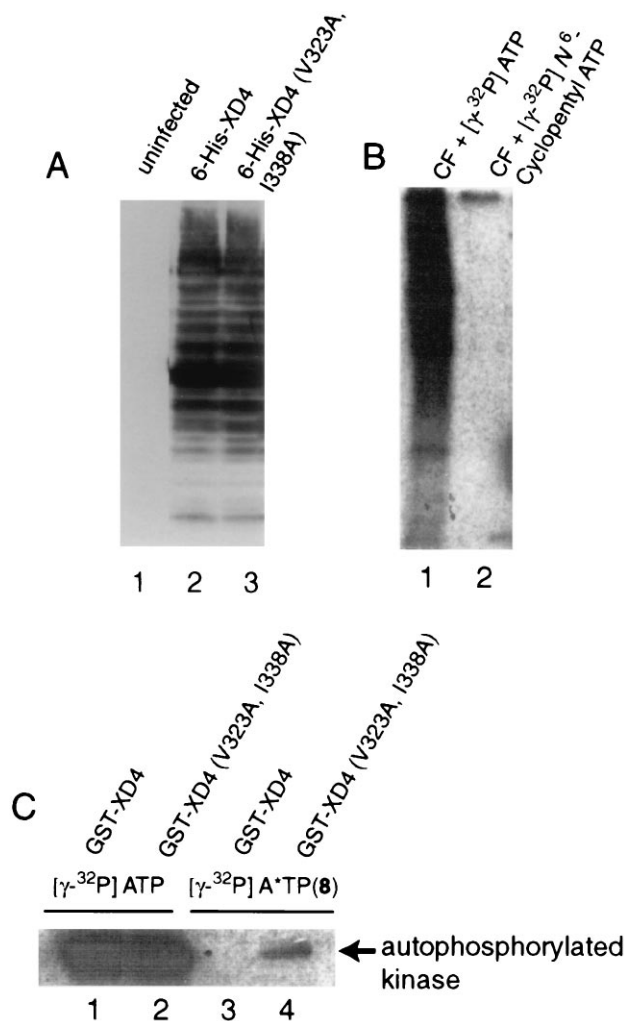


FIG. 5. (A) Lanes: 1, anti-phosphotyrosine protein blot of cell lysates (2 × 10<sup>5</sup> cell equivalents/lane) from uninfected Sf9 insect cells; 2, Sf9 cells expressing 6-His-XD4; and 3, Sf9 cells expressing 6-His-XD4(V323A, I338A). (B) Autoradiogram showing the level of phosphorylation in hypotonically lysed murine lymphocytes with [ $\gamma$ -<sup>32</sup>P]ATP (lane 1) and [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl)ATP (lane 2). (C) Autoradiogram showing [ $\gamma$ -<sup>32</sup>P]ATP-dependent autophosphorylation of GST-XD4 (lane 1) or GST-XD4(V323A, I338A) (lane 2) and [ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-(cyclopentyl) ATP-dependent phosphorylation of GST-XD4 (lane 3) or GST-XD4(V323A, I338A) phosphorylation (lane 4).

bulk of cellular phosphorylation (only 0.03% of all phosphoamino acids are tyrosine) (42) accept the synthetic substrate. To establish that [ $\gamma$ - $^{32}$ P]N<sup>6</sup>-(cyclopentyl) ATP is a dead substrate for all wild-type cellular kinases, *in vitro* kinase reactions with [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]N<sup>6</sup>-(cyclopentyl) ATP were performed with murine lymphocyte lysates (Fig. 5B). There are no radiolabeled phosphoproteins in the cell lysate after addition of [ $\gamma$ - $^{32}$ P]N<sup>6</sup>-(cyclopentyl) ATP, confirming the true orthogonal nature of N<sup>6</sup>-(cyclopentyl) ATP with respect to all wild-type protein kinases. The same result was found when *in vitro* kinase reactions with [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]N<sup>6</sup>-(cyclopentyl) ATP and NIH 3T3 cell lysates were used instead of freshly isolated murine lymphocytes (not shown). In principle, the ability to follow one protein kinase's activity in the presence of all other cellular kinases would allow for the identification of the direct kinase targets in a particular cell type. Attempts to trace the direct substrates of XD4(V323A, I338A) in Sf9 insect cells showed primarily XD4 autophosphorylation in *in vitro* kinase reactions (not shown). The predominant autophosphorylation apparent in this assay may be due to the lack of SH3 and SH2 targeting domains in XD4 or to the lack of good v-Src substrates in the heterologous insect cells. We are currently introducing full length v-Src containing the V323A, I338A mutations into NIH 3T3 cells to determine the direct substrates of v-Src in this cell type using membrane permeabilization (43) and a cell permeable form of A\*TP to introduce [ $\gamma$ - $^{32}$ P]A\*TP into fibroblasts (44).

## CONCLUSION

Our engineering efforts have provided a v-Src kinase domain that shows completely novel specificity for a synthetic nucleotide substrate analog while maintaining its wild-type specificity for tyrosine-containing peptides and proteins, thus satisfying our initial design goals. By exploiting the highly conserved nature of the ATP binding site across the kinase superfamily and the availability of structural information from other protein kinases, we were able to engineer novel substrate specificity for v-Src without any detailed structural information about v-Src itself. That we used an unrelated kinase as a blueprint for designing orthogonal ATP analogs to tag the direct cellular substrates of v-Src suggests that this approach should work for other kinases as well. If it does, then it might be possible to systematically begin to dissect the complex proximal signaling cascades controlled by cellular tyrosine kinases.

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